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Use of high density antibody arrays to validate and discover cancer serum biomarkers

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ABSTRACT

Perhaps the greatest barrier to translation of serum biomarker discoveries is the inability to evaluate putative biomarkers in high throughput validation studies. Here we report on the development, production, and implementation of a high-density antibody microarray used to evaluate large numbers of candidate ovarian cancer serum biomarkers. The platform was shown to be useful for evaluation of individual antibodies for comparative analysis, such as with disease classification, and biomarker validation and discovery. We demonstrate its performance by showing that known tumor markers behave as expected. We also identify several promising biomarkers from a candidate list and generate hypotheses to support new discovery studies.

1. Introduction

Serum protein biomarker assays may potentially play many roles in the clinical management of cancer including risk assessment, early detection, distinguishing between benign and malignant tumors, monitoring for recurrence, determining appropriate treatment, and establishing prognosis (Aebersold et al., 2005; Davis and Hanash, 2006; Hartwell et al., 2006; Vitzthum et al., 2005). Many research groups are undertaking efforts to identify putative biomarkers using genomic or proteomic strategies. Proteomic discovery approaches such as mass spectrometry can identify a large number of novel targets, even without an antibody, but their follow-up is often limited in practice to those proteins for which an antibody is currently available. Because the number of commercially available antibodies certainly exceeds the number of proteins that have been identified in serum or plasma by traditional proteomic approaches (States et al., 2006) and the number is rapidly growing, one might consider techniques which profile plasma using the growing libraries of commercial antibodies. When used in a microarray format, antibody arrays represent a cost-effective advance in precision, throughput, and protein coverage, compared to mass spectrometry-based proteomics.

We have created a high-density microarray platform that has the capacity to hold more than 18,000 binding agents.

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The goal was to create a platform that contained several libraries of antibodies of particular interest to one or more disease sites. We then probed these arrays using serum samples from ovarian cancer cases and controls in order to identify high quality candidate biomarkers and to evaluate putative biomarker candidates. Arrays were probed with cancer or control sera depleted of its most abundant protein and labeled with Cy5 (red) along with depleted reference serum labeled with Cy3 (green), yielding data directly analogous to two channel genomic arrays. Variations on this approach have been described by other groups using antibody array technology (Angenendt et al., 2002; Bereczki et al., 2007; Bi et al., 2007; Gu et al., 2006; Haab et al., 2001; Han et al., 2006; Ko et al., 2005; MacBeath and Schreiber, 2000; Miller et al., 2003; Orchekowski et al., 2005; Peluso et al., 2003; Sreekumar et al., 2001; Steinhauer et al., 2006; Usui-Aoki et al., 2007; Wacker et al., 2004). The benefits of using microarray platforms are that they permit a cost effective approach to comparative proteomic studies of plasma using a single antibody, they utilize array spotting equipment available in many research facilities, and they utilize data analysis tools commonly used in genomic array analysis. This manuscript builds on the success of previous contributions, many of which provided extensive characterization of the performance of antibody array technologies. We provide a demonstration of their performance when used in a clinical proteomics discovery application. The performance of the platform with clinical samples and endogenous protein levels is shown to be sensitive enough to identify known biomarkers.

Here we demonstrate the overall validity of this platform to profile the human serum proteome. The current array version contains 320 full-length antibodies (monoclonal or polyclonal), each printed in triplicate. Arrays were probed with serum from 31 ovarian cancer cases and 34 matched controls. The antibodies were pre-selected to represent three groups: Group 1 contained 12 antibodies to three previously validated biomarkers including CA125 ($n = 8$; Bast et al., 1981) HE4 ($n = 2$; also known as WFDC2; Hellstrom et al., 2003), and mesothelin ($n = 2$; also known as SMR; McIntosh et al., 2004); Group 2 contained a total of 38 candidate biomarkers in need of further validation that were identified in our previous discovery studies or in the literature (Biade et al., 2006; Bratt, 2000; Davidson et al., 2006; Frank and Carter, 2004; Lau and Chiu, 2007; Lim et al., 2007; Liu et al., 2006; Moubayed et al., 2007; Treiber et al., 2006; Witton et al., 2003); and Group 3 was a discovery set of 270 antibodies to cytokines, angiogenic factors, cancer antigens, differentiation markers, oncoproteins, and signaling molecules, none of which had a priori expectations of being ovarian cancer biomarkers. A complete list is contained as supplementary material. A total of 90 antibodies from this third group were also pre-specified to be one of three subgroups of interest, including 19 regulated by hypoxia, 61 that are part of the mitogen-activated protein kinase (MAPK) pathway, and 10 related to the phosphatidyl inositol 3-kinase (PI3K) pathway. As expected, Group 1 antibodies performed the best, followed by groups 2 and 3. In addition to validating individual antibodies, we were able to establish that the subgroup of MAPK proteins might as a group (Subramanian et al., 2005) contain a rich source of biomarkers.

2. Results

Our platform was able to simultaneously determine the relative abundances of over 300 proteins with reproducibility both within and between arrays sufficient to confirm the validated Group 1 biomarkers, to evaluate markers from Group 2, and to generate hypotheses regarding potential areas for new biomarker discoveries among the Group 3 antibodies. A representative array is shown in Figure 1 to illustrate the high quality spot-morphology and signal-to-noise ratio achieved by our fabrication methods.

The reproducibility of antibody printing was evaluated within-array and between-array to ensure that antibody-to-antibody comparisons for each serum sample were reliable. The intra-array reproducibility was evaluated by computing from the triplicate measurements the estimated coefficient of variation (CV) of the Red to Green ratio, $M = \text{Log(Red)} - \text{Log(Green)}$, and relating this observed CV to the overall signal intensity of the spot ($A = (\log R + \log G)/2$). A scatter plot relating the CV to A for all 320 antibodies from a randomly selected array is shown in Figure 2A. As could be expected, the platform was found to reproduce ratios whose variation partially depended on the overall signal intensity (more intense signals having lower CV), yet the majority of array features displayed a CV under 10%; specifically, across the array at the 1st, 2nd, and 3rd quartile of the average of three antibody signal intensities the antibody CV was 7.93%, 9.14%, and 3.90% respectively (Figure 2B).

Many factors might contribute to day-to-day variation in array reproducibility (e.g., varying Cy-labeling efficiencies, different ozone levels across days, daily variations in array blocking, serum hybridization, washing, or scanning parameters). To evaluate these issues in aggregate, pairs of arrays were probed on different days but with the same set of case and reference sera, each serum sample prepared (i.e., depleted of abundant proteins and Cy-labeled) on different days.

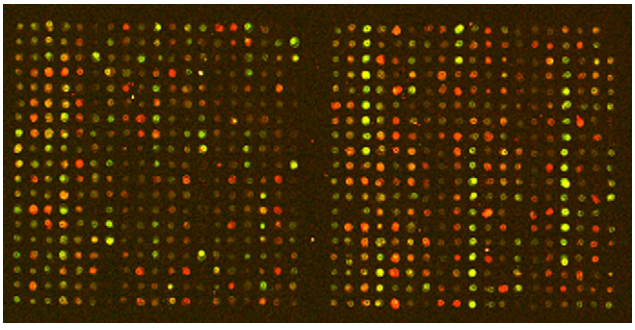

Figure 1 – Detailed view of a portion of a representative array used in this study. Each array was simultaneously challenged with serum from a unique case or a control labeled with Cy5 (red), and an equal weight (approximately 500 μg) of serum from a common reference pool (consisting of pooled normal sera) labeled with Cy3 (green). The full array consisted of 320 antibodies printed in triplicate along with various control features. Red spots indicate preferential binding of antigen in case or control serum; green features indicate preferential binding of antigen in reference sera; yellow features indicate bound antigen was equally concentrated (roughly) in both sera samples.

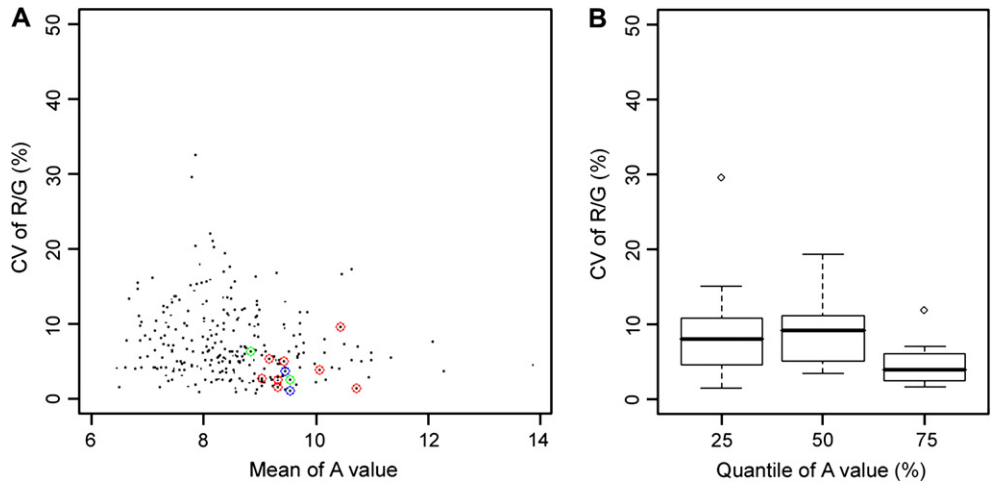

Figure 2 – Intra-array data reproducibility. Each antibody ($n = 320$) was printed in triplicate on the arrays used in this study. For each antibody feature ($n = 960$), antigen binding was measured in both red and green channels. A. The coefficient of variation of the red/green ratio across the three replicate features for each antibody was plotted as a function of the average signal intensity of those three features. Known biomarkers displayed low variation and high signal intensity (CA125 circled red, HE4 blue, and mesothelin green). B. Percent variation of R/G signal across antibody triplicates at the 1st, 2nd, and 3rd quartile of signal intensities (7.93, 9.14, and 3.90, respectively) reveals that variation decreases as signal increases, and averages below 10% across the array.

Following normalization and processing (see Section 4), we evaluated the correlation between the red and green channels across the arrays. Across eight such paired comparisons the correlations averaged 0.853, with a standard deviation 0.032. To demonstrate that this reproducibility was sufficient to identify and confirm biomarkers, supervised classification of the samples was performed, as described in Section 4. Several biomarkers were identified as significant classifiers of cancer versus control by having a low p-value and low false discovery rate (FDR, or q-value) (Storey, 2003; Storey et al., 2004). The list of the top 20 antibodies and their ranking (p-value and q-value) is shown in Table 1. The rankings for all 320 antibodies are contained in supplementary material. Figure 3A shows a histogram plot of the 320 p-values from the array, showing that overall the array is enriched for low (significant) p-values.

Among the top-performing array features were the 12 antibodies to the known ovarian cancer biomarkers CA125, mesothelin, and HE4. Each of these markers were significant classifiers of disease status on their own based on their FDR and p-value, and also as a group these markers were significantly elevated compared to the entire array. Figure 3B shows a scatter plot that illustrates individual features' ability to

Table 1 – List of the top 20 performing antibodies, ranked by ability to predict case/control status (p-value). Also presented are estimates for the magnitude and direction (log (OR)), signal intensity (A value), and false discovery rate (q-value)

Rank	Antibody	A-value	Log(OR)	p-value	q-value
1	phosohoS6	8.05565	−7.2552138	0.000818	0.21368
2	HIF-3alpha	7.90614	4.388068	0.001498	0.21368
3	Mesothelin (a)	9.65282	4.915811	0.002135	0.21368
4	CA125 (a)	9.21372	4.5075228	0.002735	0.21368
5	MEK kinase-3	8.22984	4.2112121	0.002873	0.21368
6	CA125 (b)	9.34961	3.460298	0.003952	0.21368
7	ZPK	8.3765	4.6109141	0.005129	0.21368
8	Sin3A	10.4838	−2.1333485	0.005871	0.21368
9	Keratin 8	9.47365	3.7085291	0.008377	0.24651
10	CA125 (c)	10.1828	2.5294775	0.009803	0.24651
11	Rb	9.33374	2.728865	0.012195	0.24651
12	Apolipoprotein-10A8	9.34499	3.5609945	0.012555	0.24651
13	Psoriasin	10.5972	1.7297947	0.015522	0.24651
14	CAIX	8.44885	1.6281351	0.016094	0.24651
15	Phospho-MSK1	9.29204	−1.6858844	0.016733	0.24651
16	MKP-3	8.49496	2.268331	0.017144	0.24651
17	PEBP1	9.3479	3.1762275	0.019775	0.24651
18	HIF-1alpha	9.66678	1.2047112	0.022103	0.24651
19	Prolactin	9.35183	4.1613284	0.023634	0.24651
20	MEK kinase-4	8.09536	3.0454727	0.025347	0.24651

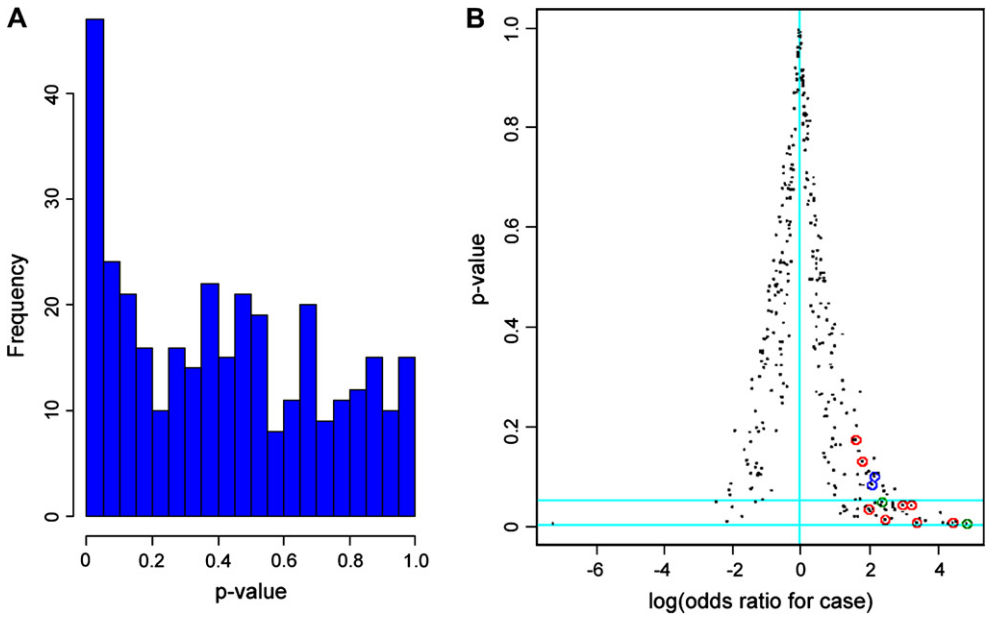

Figure 3 – Ability of arrays in this study to identify ovarian case ($n = 31$) versus control ($n = 34$) sera. Logistic regression was used to estimate each antibody's ability to predict disease. A. The probability score for each antibody (p-value determined by Wilcoxon sign-rank testing), displayed as a function of the number of antibodies with that score. The leftward skew indicates that this array was enriched for antibodies capable of identifying case/control status with high probability. B. The p-value of each antibody displayed as a function of the log of the odds ratio for case. Those data points below the horizontal aqua line indicate antibodies capable of differentiating case and control sera with statistical significance ($p = 0.05$ or less); data points to the right of the vertical aqua line represent antibodies that preferentially bind antigens in case sera, while data points to the left of this line represent antibodies that preferentially bind antigens in control sera. All antibodies to known biomarkers preferentially recognized case sera, most (8/12) with statistical significance (red circles = CA125, green = mesothelin, and blue = HE4).

distinguish case from control (expressed as the log odds ratio of their ability to predict case status versus p-value). As indicated by the color-encircled data points (red = CA125, blue = HE4 and green = mesothelin), all twelve of these antibodies were identified as good predictors of case status.

The average rank of all 12 antibodies was 41 out of 320, and all placed within the top 30% of antibodies on the array ($p < 0.001$ Wilcoxon rank sum test). These antibodies also had low levels of variation among triplicate spots compared to the remainder of the array (mean CV 3.67%) and had higher signal intensity (average A value 9.58). Taken together, these data indicate our platform is efficient and reliable to evaluate in high throughput the case/control status of human serum using validated biomarkers of disease.

Among these 12 antibodies, the top performing was mesothelin. However, the differences between these top-performing markers was not statistically significant, so although one can conclude that each biomarker is a significant classifier of disease status, we cannot with these data conclude that one marker is superior to another in this study. The significance and effect size presented here are based on odds ratios, which are an alternate effect size for classification rules, and which are appropriate when, as with our analysis, covariate adjustments are made (Pepe et al., 2004). These markers were also evaluated for their Receiver Operating Characteristic (ROC) curve and their area under the ROC curve (AUC) based on their un-normalized values, and we found these analyses concordant with the odds ratio in terms of direction and approximate magnitude. For example, the AUCs for the eight CA125 antibodies averaged 0.65 (st. dev. 0.059), the two for mesothelin averaged 0.67 (st. dev. 0.064) and the two for He4 ($n = 2$) averaged 0.61 (st. dev. 0.026).

These findings demonstrate that the performance of previously validated antibodies are recapitulated on this platform. We also used this platform to evaluate a number of other putative biomarker candidates. This second group of antibodies, to biomarker candidates in need of validation, were overall found to be ranked higher compared to the remainder of the array ($p < 0.001$, Wilcoxon-rank sum test) with an average ranking of 86.1 out of 320. This suggests that this group of antibodies contains one or more targets with the ability to classify case or controls. The complete list of these putative biomarkers is provided in the supplementary table as indicated.

The final group of antibodies on this array was a discovery set, assembled from many classes of proteins including diverse signaling pathway members but without any a priori expectation of being a biomarker for any single antibody. As expected, this group performed worst of all three. Most of these antibodies do not reliably classify case versus control status. Given the relatively small sample size of our serum set, one could not expect to make a definitive discovery of specific novel biomarkers from this group using this study alone. However, to increase power one might consider interrogating groups of biomarkers from specific pathways to generate hypotheses regarding the source for new markers (Subramanian et al., 2005). Of 270 total antibodies from this discovery set, the subgroup defined by its relation to MAPK ($n = 61$) was shown

to be an overall significant predictor of case versus control status compared to the remainder of the set ($p < 0.001$ Wilcoxon rank sum test; average rank 101.5).

3. Discussion

Antibodies are nature's best affinity capture reagents, and are perfectly suited for characterization of complex proteomes such as human serum due to their high affinity and specificity. Here, we report our ability to successfully immobilize 320 unique antibodies in high density on a single microarray, with functionality suitable for biomarker validation and discovery (as indicated by robust and reproducible antigen binding). These microarrays were also suited for incubation with small volumes of serum (~50 μl total starting material per interrogated sample) and so were practically feasible for use with rare specimens.

To validate the platform, we included on the array several antibodies to biomarkers known to have the capacity to classify ovarian cancer from control specimens. The intent was to profile and rank all antibodies on the array and confirm the overall approach by measuring the performance of these known markers. Importantly, our array succeeded in confirming known markers. This confirmation is even more striking given the sample size and our use of multiple and heterogeneous controls (i.e., from women with benign disease and women undergoing surgery), which may potentially decrease the power of our array compared to using a homogenous control group. Of the 34 controls used in this study, 16 were healthy, 8 were surgical normal, and 10 had benign conditions of the ovary.

The major purpose of such a high density array is the screening of a large number of antibodies to determine whether their targets can classify subject samples. However, there are several issues to consider when interpreting the results. In our format, one should take effort to further identify or verify the identity of the actual analyte of the best performing antibodies as they could be different than the manufacturer's putative characterization. In addition, caution must be exercised in asserting that the expected target of an antibody is not a biomarker. Compared to an ELISA format with enzymatic amplification, one could expect that sensitivity may decline and a non-validated biomarker could simply have fallen under the assay's detection limit in this format. However, it should be noted that our fractionation/concentration procedures facilitate detectable protein concentrations for low-abundant proteins such as IL1β for which we could detect both endogenous levels of protein as well as a 0.087 (SD = 0.022) increase in M value when 2 pg and a 0.163 (SD = 0.1384) increase when 5 pg of IL1β was 'spiked-in' to a sample compared to the sample alone (increase in IL1β was significantly different, $p < 0.0001$, Wilcoxon test). We also performed conventional ELISA (2 antibody) analyses of the CA125 and mesothelin levels in these same serum samples and both correlated with results from our array ($R^2 = 0.65$ and 0.73 respectively, both $p < 0.0001$).

Certain high ranking members of the validation set may now be ready for larger studies required to assess their sensitivity and specificity, and these tests may reliably be conducted using our platform with larger sample sets. Although, as expected, few members of the discovery library showed classification potential on an individual level, several might deserve further study. Among these are members of the mitogen activated protein kinase (MAPK) cascade, long known to play a role in carcinogenesis (reviewed in Hanahan and Weinberg, 2000). Discovery of cytoplasmic or nuclear proteins as putative serum biomarkers could indicate sufficient apoptosis/necrosis of early neoplastic cells, or could indicate that the antibody was binding other proteins with similar epitopes. Interestingly, three other highly ranked members of the discovery set belonged to the family of proteins involved in hypoxic response. Members of this family of proteins have previously been implicated in ovarian carcinoma (Birner et al., 2001; Chi et al., 2006) and also certainly warrant further investigation.

Antibody array technology has been developing for over a decade, and has successfully been applied to the discovery of potential biomarkers. Mor and colleagues utilized proprietary antibody microarray technology to analyze 169 total proteins from the sera of 18 untreated EOC patients and 28 healthy controls (Mor et al., 2005). Thirty-five proteins were found to be differentially expressed, and a validation study involving 100 cases and 106 controls showed that four of them – leptin, prolactin, osteopontin, and insulin-like growth factor II- could detect ovarian cancer with 95% sensitivity and 95% specificity. Like other research groups, Mor and colleagues utilized antibody arrays for discovery of putative biomarkers; validation was achieved using standard, low-throughput means. Furthermore, commercial arrays like those used by Mor et al. come at significant expense and provide little flexibility to the researcher in selecting antibodies during fabrication.

The advantage of in-house fabricated arrays includes the ability of researchers to curate their own antibodies of interest to one or multiple diseases. Additionally, costs may be better managed, and issues relating to data ownership are avoided. Indeed, our group is currently fabricating an array with recombinant antibody fragments, and full-length antibodies of interest in ovarian, breast, and colon cancers, allowing researchers to evaluate biomarkers for diagnostic utility and disease specificity. The sensitivity and specificity of an array are dependent upon the antibodies chosen, as well as the disease sera probed, and can be optimized and changed over time as well-performing antibodies are added and retained, and poorly performing antibodies removed. The ability to support high throughput validation and discovery using only one antibody could be a promising approach for accelerating progress in translational research for ovarian cancer.

4. Experimental procedures

4.1. *Fabrication and printing of arrays*

Buffers, protocols, slide surfaces and techniques were modified from DNA array-based technology and existing antibody array-based literature. Triplicate features of each antibody were printed. Previous array experiments confirmed that spatial separation resulted in no loss of reproducibility among

the three common features (data not shown). Antibodies were arrayed at the DNA Array Facility at FHCRC (J. Delrow, director). Antibodies were diluted in PBS (50 mM potassium phosphate, pH 7.2, 150 mM NaCl) to a final concentration of 0.20–0.25 mg/ml. This mixture was combined with an equal volume (4 μl) of 2× Protein Printing Buffer (Telechem); nanoliter volume of this mixture was transferred from a microtiter plate onto Nexturian hydrogel-coated glass slides. Coupling occurred at room temperature overnight.

4.2. Selection and description of Sera

Cancer and control sera were selected from a repository collected as part of several human subjects approved research projects funded by the National Cancer Institute (NCI), but collected under the same protocols (Hellstrom et al., 2003; McIntosh et al., 2004, 2007). All specimens used in this study were collected between 1 July 2004 and 10 May 2006, and include pre-treatment serum samples from ovarian cancer cases ($n = 31$) diagnosed at the time of surgery with primary serous ovarian cancer located in the ovary, fallopian tube, or peritoneum, at various stages of disease: stage IA ($n = 2$), stage IC ($n = 2$), stage IIA ($n = 1$), stage IIC ($n = 1$), stage IIIB ($n = 2$), stage IIIC ($n = 18$), stage IVA ($n = 4$), and stage IVB ($n = 1$). Controls ($n = 34$) were a heterogeneous collection of three types; healthy controls were samples collected from healthy women attending regular mammography screening exams ($n = 16$), surgical controls were women who underwent surgery for non-ovarian related gynecologic conditions and who had histologically normal ovaries ($n = 8$), and benign controls were women with benign ovarian cysts or tumors ($n = 10$). Sample processing protocols for all specimens were identical. All surgical samples (case, surgical control, and benign) were collected prior to surgery and chemotherapy under the same collection protocols.

Controls were selected using propensity score matching so that case and control groups were balanced with respect to age, risk status, and collection date within 3 years. The age at collection ranges between 52 and 87 (mean 61.6; standard error 9.8). The distribution of age at collection for controls was very similar to that of cases (range 42–86 with mean 60.9 and standard error 10.6).

4.3. Sera treatment

It is generally accepted (although not quite universally) in the proteomics community that one should deplete abundant serum proteins prior to interrogation using discovery platforms and that these depletion schemes affect each protein differently (Whiteaker et al., 2007). Although many depletion schemes are available for this analysis we chose to deplete using cibacron blue due to its suitability for high throughput use and our relatively simple need to only reduce the concentration of albumin. Obviously, a limited number of other proteins also bind to cibacron blue so their concentration will be reduced and evaluation as biomarkers affected. Specifically sera was incubated with Cibacron Blue 3GA immobilized on agarose beads (Sigma C-1535, St. Louis, MO) to remove >95% of serum albumin. Washed beads (in 10 mM Tris, pH 8.0) were added at a 1:1 ratio (v/v) to serum diluted 1:10 in 10 mM Tris, pH 8.0, and platform rotated at room temperature for 5 min. The bead-serum slurry was centrifuged at 2000 rpm for one minute, and the serum-supernatant saved. Serum was then concentrated to its original volume (not to dryness) by speed-vacuum centrifugation, measured for total protein concentration by BCA assay (Pierce Biotechnology, Rockford, IL), and normalized prior to challenging the arrays.

4.4. Fluorescent labeling of serum proteins

Detecting biomarkers on the arrays requires direct incorporation of a tag or label. Serum protein was labeled with the amine reactive dyes Cy3 and Cy5 (GE Amersham, Piscataway, NJ) according to the manufacturer's instructions. Unincorporated dye was removed by Microcon centrifugation dialysis (10,000 MW cutoff; Millipore Corp.). For this study, case and control sera were always labeled with Cy5, and separately challenged against an array alongside Cy3-labelled reference sera (a common pool of normal serum used as reference for all samples). Approximately 500 μg of total serum protein was labeled according to the manufacturers instructions.

4.5. Array blocking

Printed array slides were placed in a slide rack and washed vigorously in phosphate buffered saline-0.01% Tween 20 (PBST) by dunking 30 times, washed twice in fresh water by dunking 30 times, then placed in ethanolamine block solution (0.3% ethanolamine, 0.05 M sodium borate pH 8.0) and dunked 30 times. After 2–3 h incubation (orbital shaker, 80 rpm) in block solution, the slides were dunked 30 times in PBST, twice in fresh water, and then dried immediately by spinning $1000 \times g$ for 5 min at room temperature. Dried slides were immediately challenged with labeled sera.

4.6. Challenging arrays with sera

Array slides were placed on a level surface and 65 μl of labeled sera was applied by micropipette. Coverslips were immediately placed on the slides over the array. Slides were covered with aluminum foil to prevent exposure to light, and were incubated with sera for approximately 2 h. Following incubation, slides were placed into a slide rack submerged in PBST such that the coverslip fell to the bottom of the dish. Slides were rotated on orbital shaker (80 rpm) for 5 min. The slide rack was then moved to a fresh dish of PBST, dunked vigorously 30 times, and shaken (80 rpm, orbital shaker) for 5 min. This entire wash step (i.e., dunking and shaking) was then repeated with PBS, followed by two washes in fresh water (dunking 30 times only). Slides were immediately dried by centrifugation at $1000 \times g$ for 5 min at room temperature. Slides were then scanned in a GenePix 4000B microarray scanner (Axon Instruments).

4.7. Array analysis and normalization

Array data contains a format identical to two-channel gene expression arrays and analysis proceeds analogously. Briefly, technical sources of variation are normalized using loess procedures developed for micro-arrays (Smyth and Speed, 2003).

When choosing our normalization procedure for the array we evaluated three different normalization approaches, including variations of VSN (variance stabilization normalization: globally or on print-tip), and loess methods (on print-tip, plate and antibody type). Each method was evaluated on their ability to reduce variation across multiple independent days of control-to-control and case-to-case comparisons. For each antibody, fold-change of signal (case or control compared to reference channel) was calculated as log Rc/Gc; where Rc is red corrected and Gc is green corrected (using normexp background correction method developed by Smyth, 2005). Following normalization triplicate spots were summarized using their median. Classification was performed using logistic regression predicting case status using *M* value adjusted for operator, batch, and their interaction effects. The *p*-value corresponding to the coefficient of the *M* value was used for ranking the antibodies. Assignment of antibodies to the 3 groups (biomarkers, validation and discovery) was done prior to data analysis. Assignment of the discovery antibodies to the MAPK, PI3K, and hypoxic subgroups was difficult as many of the pathways are expansive; we recognize that many antibodies to proteins may have been missed or even misassigned.

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Appendix I. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molonc.2007.08.004.

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